

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

08/24/98
jc408 U.S. PRO
09/138735

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Attorney Docket No.: WPB 36400B

Date: August 24, 1998

jc588 U.S. PRO
09/138735
08/24/98

BOX PATENT APPLICATION

Assistant Commissioner for Patents
Washington, D.C. 20231

CONTINUING APPLICATION TRANSMITTAL RULE 1.53(b)

Sir:

Transmitted herewith for filing under 37 C.F.R. §1.53(b) is a

☐ Continuation

☒ Divisional

☐ Continuation-in-Part

application of prior pending Application No. 08/480,917, filed June 7, 1995,

For (Title): TRYPANOSOMA CRUZI ANTIGEN, GENE ENCODING THEREFOR AND METHODS OF DETECTING AND TREATING CHAGAS DISEASE

By (Inventors): Glaucia PARANHOS-BACCALA; Mylene LESENECHAL and Michel JOLIVET

1. ☒ A Declaration and Power of Attorney is attached. The attached Declaration and Power of Attorney is:
 - ☒ a. A copy of the Declaration and Power of Attorney from the parent application. (Used with the same or fewer inventors and (a) a copy of the prior application or (b) a revised, reformatted or edited version of the prior application that does not contain new matter.)
 - ☐ b. A new Declaration and Power of Attorney. (Used with the same, fewer or additional inventors and (a) a copy of the prior application, (b) a revised, reformatted or edited version of the prior application that does not contain new matter, or (c) a new specification.)
2. ☒ The filing fee is calculated below:

CLAIMS IN THE APPLICATION AFTER ENTRY OF ANY PRELIMINARY AMENDMENT NOTED BELOW

FOR:	NO. FILED	NO. EXTRA
BASIC FEE		
TOTAL CLAIMS	20 - 20	= 0*
INDEP CLAIMS	1 - 3	= 0*
<input type="checkbox"/> MULTIPLE DEPENDENT CLAIMS PRESENTED		

* If the difference is less than zero, enter "0".

SMALL ENTITY

RATE	FEE
	\$ 395
x 11 =	\$
x 41 =	\$
+135 =	\$
TOTAL	\$

OR
OR
OR
OR
OR
OR

OTHER THAN A SMALL ENTITY

RATE	FEE
	\$ 790
x 22	\$ ----
x 82	\$ ----
+270	\$ ----
TOTAL	\$ 790

3. ☒ Check No. 61059 in the amount of \$790.00 to cover the filing fee is attached. The Commissioner is hereby authorized to charge any other fees that may be required to complete this filing, or to credit any overpayment, to Deposit Account No. 15-0461. Two duplicate copies of this sheet are attached.
4. ☐ Cancel claims _____ of the application before calculating the filing fee. At least one independent claim is retained for filing purposes.

DEPOSIT ACCOUNT USE AUTHORIZATION

Please grant any extension
necessary for entry;
Charge any fee due to our
Deposit Account No. 15-0461

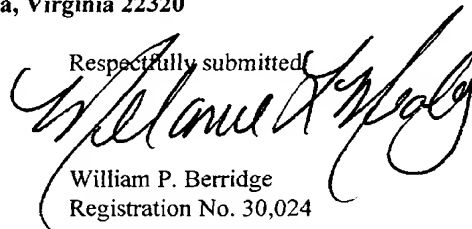
09/138735 08/24/98

5. ☒ Amend the specification by inserting before the first line the sentence:
--This is a ☐ Continuation ☒ Division ☐ Continuation-in-Part of Application No. 08/480,917 filed June 7, 1995. The entire disclosure of the prior application(s) is hereby incorporated by reference herein in its entirety.--
6. ☒ Formal drawings (Figs. 1-2) are attached.
7. ☒ Priority of foreign application No. 94 10132 filed August 12, 1994 in France is claimed under 35 U.S.C. §119 and/or §365(b).
☒ The certified copy was filed in prior Application No. 08/480,917 on September 7, 1995.
☐ A certified copy of the above foreign application(s) is filed herewith.
8. ☐ Priority of U.S. Provisional Application(s) No. _____ filed _____ is claimed under 35 U.S.C. §119.
☐ Amend the specification by inserting before the first line the sentence:
--This nonprovisional application claims the benefit of U.S. Provisional Application(s) No. _____ filed _____--
9. ☒ The prior application is assigned of record to BIO MERIEUX recorded at Reel 7626, Frame 0182.
10. ☐ This application is filed by fewer than all the inventors named in the prior application (37 C.F.R. §1.53(d)(4)). Delete the following inventor(s) named in the prior application:

11. ☒ A Preliminary Amendment is attached. Claims added by this Amendment are properly numbered consecutively beginning with the number next following the highest numbered claim in the application.
12. ☒ An Information Disclosure Statement is attached.
13. ☐ Small entity status:
☐ a. A small entity statement is attached.
☐ b. A small entity statement was filed in the parent application and such status is still proper and desired.
☐ c. Small entity status is no longer claimed.
14. ☐ Other: _____
15. ☒ The power of attorney in the application is to James A. Oliff, Registration No. 27,075, William P. Berridge, Registration No. 30,024, Kirk M. Hudson, Registration No. 27,562, Thomas J. Pardini, Registration No. 30,411, Edward P. Walker, Registration No. 31,450, Robert A. Miller, Registration No. 32,771 and/or Mario A. Costantino, Registration No. 33,565.
☒ a. The power appears in the attached Declaration and Power of Attorney.
☐ b. Since the power does not appear in the attached Declaration and Power of Attorney, a substitute Power of Attorney is also attached.
16. ☒ Address all future communications to:

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Respectfully submitted,


William P. Berridge
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WPB:CLC/jca

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Glaucia PARANHOS-BACCALA; Mylene
LESENECHAL and Michel JOLIVET

Prior Group Art Unit: 1641

Application No.: Rule 1.53(b) Div. Appln. of U.S.
Appln. No. 08/480,917, filed June 7, 1995

Prior Examiner: J. Shaver

Filed: August 24, 1998

Docket No.: WPB 36400B

For: TRYPANOSOMA CRUZI ANTIGEN, GENE ENCODING THEREFOR AND
METHODS OF DETECTING AND TREATING CHAGAS DISEASE

PRELIMINARY AMENDMENT

Assistant Commissioner of Patents
Washington, D. C. 20231

Sir:

Prior to initial examination, please amend the above-identified application as follows:

IN THE TITLE:

Page 1, above the first line, insert the following title: --TRYPANOSOMA CRUZI
ANTIGEN, GENE ENCODING THEREFOR AND METHODS OF DETECTING AND
TREATING CHAGAS DISEASE.--

IN THE SPECIFICATION:

Please amend the specification as follows:

Page 1, before line 1, insert:

--FIELD OF THE INVENTION--;

09138735 "082498
064280" 9225160

between lines 5 and 6, insert:

--BACKGROUND OF THE INVENTION--.

Page 2, line 1, change "epi-mastigote" to --epimastigote--;

line 26, change "tests, such as" to --tests include--.

Page 3, line 29, change "where" to --were--.

Page 4, line 1, change "sero-logical" to --serological--;

line 4, change "anti-gen" to --antigen--;

between lines 6 and 7, insert:

--SUMMARY OF THE INVENTION--;

between lines 22 and 23, insert:

--DETAILED DESCRIPTION OF THE INVENTION--;

line 26, change "No.1," to --NO:1,--;

line 28, change "No.1," to --NO:1,--.

Page 5, line 5, change "No.1" to --NO:1--.

Page 7, line 10, change "No.1" to --NO:1,--;

line 12, change "No.1" to --NO:1, and--;

line 13, delete "and" (first occurrence);

line 14, after "2207" insert --of SEQ ID NO:1--;

line 26, change "No.2." to --NO:2.--.

Page 8, line 3, change "No.2," to --NO:2,--.

Page 9, line 4, change "(E" to --(E.--.

Page 10, line 7, change "No.2." to --NO:2.--.

Page 13, line 30, change "under-stood" to --understood--.

Page 15, line 19, change "No.1," to --NO:1,--;

line 24, change "No.1," to --NO:1,--.

Page 16, line 6, change "No.1." to --NO:1.--;

line 29, change "techniques," to --technique,--;

line 30, after "(14))," insert --the--;

line 32, before "Northern" insert --the--.

Page 17, line 2, after "target," insert --and the--;

line 4, change "tech-nique" to --technique--;

line 13, change "trans-lation" to --translation--;

line 14, change "pheno-mena." to --phenomena.--;

line 24, change "No.7," to --NO:7,--; change "No.8," to --NO:8,--; change
"No.9," to --NO:9,--; change "No.10," to --NO:10, and--;

line 25, change "No.12." to --NO:12.--.

Page 18, between lines 12 and 13, insert:

--DESCRIPTION OF THE FIGURES--.

Page 19, line 8, change "No.3" to --NO:3--; change "24," to --24--;

line 10, change "No.4" to --NO:4--; change "24" to --24,--;

line 25, change "No.I" to --NO:1--;

line 26, change "No.2," to --NO:2,--;

line 28, change "1825." to --1825 of SEQ ID NO:1.--;

line 30, change "No.2." to --NO:2.--.

Page 20, line 23, change "For that, microtiter" to --Microtiter--;

line 24, change "name), nunc)" to --name))--;

line 30, delete "entire";

line 31, change "react" to --reacts--.

Page 21, line 31, change "Tc 50" to --Tc50--.

Page 22, line 17, change "No.5" to --NO:5--;

line 18, change "No.6" to --NO:6--;

line 20, change "No.5" to --NO:5--;

line 22, change "No.6" to --NO:6--;

line 26, change "No.1," to --NO:1,--.

Page 23, line 18, change "No.7" to --NO:7--;

line 19, change "No.8" to --NO:8--;

line 21, change "No.7" to --NO:7--;

line 25, change "No.8" to --NO:8--;

line 28, change "No.1," to --NO:1,--.

Page 24, line 3, change "No.1" to --NO:1--;

line 9, change "No.9 :" to --NO:9:--;

line 11, change "No.10:" to --NO:10:--;

line 13, change "No.9" to --NO:9--;

line 16, change "No.1." to --NO:1.--;

line 17, change "No.10" to --NO:10--;

line 20, change "No.10," to --NO:10--;

line 25, change "No.1" to --NO:1--.

Page 25, line 1, change "No.11" to --NO:11--;
line 7, change "No.12:" to --NO:12:--;
line 9, change "No.13:" to --NO:13:--;
line 11, change "No.12" to --NO:12--;
line 15, change "No.11" to --NO:11--;
line 17, change "No.11." to --NO:11.--;
line 19, change "des-cribed," to --described,--;
line 22, change "No.1" to --NO:1--.

Page 37, line 23, change "1426" to --426-428--.

Page 38, line 21, change "386-389" to --836-839--.

REMARKS

By this Preliminary Amendment, the specification has been amended to place the application in better condition for initial examination and allowance. Claims 1-20 are pending.

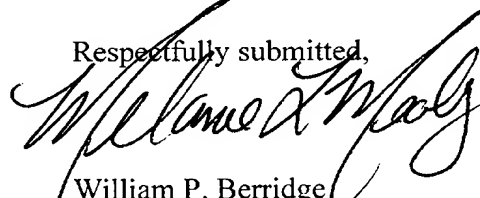
The computer readable form of the Sequence Listing in this application is identical with that filed in Application No. 08/480,917, filed on June 7, 1995. In accordance with 37 C.F.R. §1.821(e), please use the computer readable form filed in that application as the computer readable form for the instant application. It is understood that the U.S. Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used for the instant application.

Amino acids 321-340 were inadvertently omitted from SEQ ID NO:2 on page 28 of the French-language patent application, filed June 7, 1995, and from SEQ ID NO:2 on page 32 of the Verified Translation. The amino acid sequence identified in SEQ ID NO:2

corresponds to the nucleotide sequence identified in SEQ ID NO:1. One of ordinary skill in the art would have recognized from these disclosures that a segment of amino acids was missing from SEQ ID NO:2 underneath identification numbers 321-340 and would have readily been able to determine the omitted amino acid segment by translating from the corresponding codons in SEQ ID NO:1. Thus, no new matter is added.

Early and favorable action on the merits is respectfully requested.

Respectfully submitted,



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The subject of the present invention is a new genetic material encoding a new protein recognized by anti-Trypanosoma cruzi antisera, and it relates to the use of said gene and protein, especially for diagnostic, pharmaceutical and therapeutic purposes.

Trypanosoma cruzi is a flagellate protozoal parasite, a member of the order Kinetoplastida and of the family Trypanosomatidae, which is responsible for Chagas disease which affects naturally millions of persons, mainly in Latin America.

In vertebrate hosts, Trypanosoma cruzi is present in two forms: one which is mobile by means of its flagellum or trypomastigote and which does not divide; the other is aflagellate, or intracellular amastigote, which multiplies by binary division.

Transmission of the protozoan in man occurs through hematophagous insects of the family Reduviidae, during a blood meal followed by dejections at the site of the bite. The vector insect thus releases the infectious metacyclic trypomastigote forms which will colonize many cell types through the blood circulation. Trypanosoma cruzi infects cardiac and skeletal muscular cells, the glial cells and the cells of the mononuclear phagocytic system. After passive penetration into the host cell, the trypomastigote form of the parasite differentiates into the amastigote form, divides actively and then this is followed by a release of the trypomastigote forms, thereby causing a new cell invasion.

The insects will complete the parasitic cycle by ingesting, during a blood meal, the trypomastigote forms in the host. The latter differentiate into

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epi-mastigote forms in the vector's middle intestine and finally into the infectious metacyclic trypomastigote forms in the posterior intestine.

Two phases can be distinguished in the Chagas disease: the acute phase and the chronic phase. The acute phase occurs after a transfusional, congenital or vectorial type contamination and lasts for a few weeks. It is characterized by a large number of parasites circulating in the blood and corresponds to an exponential division of the protozoan. The acute phase is most often asymptomatic. However, in infants contaminated by their mother, the acute phase, which is marked by an acute cardiopathy, may be critical. The chronic phase may extend over many years. In some individuals, this phase is asymptomatic. On the other hand, other patients have tissue lesions in the heart or digestive type manifestations. In any case, clinical diagnosis must always be confirmed by tests for the detection either of antibodies directed against the parasitic antigens, or of the parasite itself.

This disease is becoming a worldwide problem because of the contamination through blood transfusion. It was therefore becoming essential to have available diagnostic tests which make it possible to determine the presence of the parasite in individuals. Various serological tests, such as direct agglutination, indirect immunofluorescence (IIF), complement fixation tests (CFR), ELISA tests (Enzyme Linked Immunosorbent Assay). The *Trypanosoma cruzi* antigens used for the serological tests are obtained from a total lysate of the noninfectious stage of the parasite or from partially purified protein fractions. However, these

fractions do not allow antigens to be obtained in sufficient quantity and quality for the production of a reliable serological diagnostic test. Furthermore, the complexity of the parasite and the strain-to-strain antigenic polymorphism introduce an additional difficulty in the reproducibility of the different preparations. Finally, there are many risks of cross-reactivity with other protozoa, more particularly with *Trypanosoma rangeli*, a nonpathogenic parasite, and the family *Leishmania*. Another disadvantage of these techniques is the absence of determination of the disease phase which would allow a treatment from the onset of the acute phase.

In order to solve these various problems, it was envisaged to produce a serological diagnostic kit composed of recombinant proteins which would be specific for *Trypanosoma cruzi*.

Various research groups have screened libraries for expression of *Trypanosoma cruzi* genomic DNA or complementary DNA in the vector λ gt11, using sera from patients suffering from Chagas disease. The λ gt11 phage allows the insertion of foreign DNA of a maximum size of 7Kb into the EcoR1 site localized in the lacZ gene, under the control of the lac promoter. The product obtained is a recombinant protein used with beta-galactosidase, which is inducible by IPTG (isopropyl beta-D-thiogalactoside).

Various *Trypanosoma cruzi* genes, encoding proteins recognized by the Chagasic sera were thus characterized. Among the recombinant antigens described, the H49 antigen may be mentioned (Paranhos et al., 1994 (1)). However, this antigen does not allow a

sero-logical detection sensitivity of 100% of the patients in the acute or chronic phase. It was therefore envisaged to combine the H49 antigen with the CRA anti-gen (Cytoplasmic Repetitive Antigen) (Lafaille
5 et al., (1989) (2)) but still without solving this problem.

The present inventors have identified and obtained for the first time a new genetic material encoding a new protein, recognized by anti-Trypanosoma
10 cruzi antisera, which makes it possible to overcome the abovementioned disadvantages. The genetic material may be used to produce proteins or polypeptides for the production of diagnostic tests, or for the preparation of vaccinal or pharmaceutical compositions, or may
15 itself either be used as a probe, or for the determination of specific probes which can be used in nucleic acid hybridization tests for the detection of Trypanosoma cruzi infections. Likewise, the protein or any corresponding polypeptide may be used for the pro-
20 duction of antibodies specific for the parasite, for diagnostic or passive protection purposes.

This gene was called Tc 100 by the applicant.

Consequently, the subject of the present invention is a DNA or RNA molecule consisting of at
25 least one strand comprising a nucleotide sequence represented in the identifier SEQ ID No.1, or a sequence complementary or antisense or equivalent to said sequence identified in the identifier SEQ ID No.1, and especially a sequence having, for any succession of
30 100 contiguous monomers, at least 50%, preferably at least 60%, or better still at least 85% homology with said sequence.

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Nucleotide sequence is understood to mean either a DNA strand or its complementary strand, or an RNA strand or its antisense strand or their corresponding complementary DNAs. The DNA sequence as
5 represented in the identifier SEQ ID No.1 corresponds to the messenger RNA sequence, it being understood that the thymine (T) in the DNA is replaced by a uracil (U) in the RNA.

According to the invention, two nucleotide
10 sequences are said to be equivalent in relation to each other, or in relation to a reference sequence if, functionally, the corresponding biopolymers can play essentially the same role, without being identical, with respect to the application or use considered, or in the
15 technique in which they are involved; two sequences obtained because of the natural variability, especially spontaneous mutation, of the species from which they were identified, or because of induced variability, as well as homologous sequences, homology being defined
20 below, are especially equivalent.

Variability is understood to mean any spontaneous or induced modification of a sequence, especially by substitution and/or insertion and/or deletion of nucleotides and/or of nucleotide fragments,
25 and/or extension and/or shortening of the sequence at at least one of the ends; a nonnatural variability may result from the genetic engineering techniques used; this variability may result in modifications of any starting sequence, considered as reference, and capable
30 of being expressed by a degree of homology relative to the said reference sequence.

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Homology characterizes the degree of identity of two nucleotide (or peptide) fragments compared; it is measured by the percentage identity which is especially determined by direct comparison of nucleotide (or peptide) sequences, relative to reference nucleotide (or peptide) sequences.

Any nucleotide fragment is said to be equivalent to a reference fragment if it has a nucleotide sequence which is equivalent to the reference sequence; according to the preceding definition, the following are especially equivalent to a reference nucleotide fragment:

a) any fragment capable of at least partially hybridizing with the complementary strand of the reference fragment,

b) any fragment whose alignment with the reference fragment leads to the detection of identical contiguous bases, in greater number than with any other fragment obtained from another taxonomic group,

c) any fragment resulting or capable of resulting from the natural variability of the species, from which it is obtained,

d) any fragment capable of resulting from the genetic engineering techniques applied to the reference fragment,

e) any fragment, containing at least 30 contiguous nucleotides, encoding a peptide homologous or identical to the peptide encoded by the reference fragment,

f) any fragment different from the reference fragment by insertion, deletion, substitution of at least one monomer, extension or shortening at at least

one of its ends; for example any fragment corresponding to the reference fragment flanked at at least one of its ends by a nucleotide sequence not encoding a polypeptide.

5 The invention moreover relates to DNA or RNA fragments whose nucleotide sequence is identical, complementary, antisense or equivalent to any one of the following sequences:

 - that starting at nucleotide 1232 and ending
10 at nucleotide 2207 of SEQ ID No.1

 - that starting at nucleotide 1232 and ending at nucleotide 1825 of SEQ ID No.1

 - and that starting at nucleotide 1266 and ending at nucleotide 2207,

15 and especially the DNA or RNA fragments whose sequence has, for any succession of 30 contiguous monomers, at least 50%, preferably at least 60%, or better still at least 85% homology with any one of said sequences.

20 The subject of the invention is also a protein, called Ptc100 by the applicant, having an apparent molecular mass of about 100 kDa, which is recognized by anti-Trypanosoma cruzi antisera, or an immunological equivalent of this protein, and fragments thereof. The
25 amino acid sequence of this protein is represented in the identifier sequence SEQ ID No.2.

 Immunological equivalent is understood to mean any polypeptide or peptide capable of being immunologically recognized by the antibodies directed against
30 said Ptc100 protein.

 The invention also relates to any fragment of the Ptc100 protein. A particular protein fragment has a

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sequence starting at amino acid 323 and ending at amino acid 520 of the sequence defined in the identifier SEQ ID No.2, said fragment being specifically recognized by anti-Trypanosoma cruzi antisera; the invention also
5 relates to any immunological equivalent of said fragment.

The Ptc100 protein and said protein fragments may contain modifications, especially chemical modifications, which do not alter their immunogenicity.

10 Moreover, the subject of the present invention is also an expression cassette which is functional especially in a cell derived from a prokaryotic or eukaryotic organism, and which allows the expression of DNA encoding the entire Ptc100 protein or a fragment
15 thereof, in particular of a DNA fragment as defined above, placed under the control of elements necessary for its expression; said protein and said protein fragments being recognized by anti-Trypanosoma cruzi antisera.

20 Generally, any cell derived from a prokaryotic or eukaryotic organism can be used within the framework of the present invention. Such cells are known to persons skilled in the art. By way of examples, there may be mentioned cells derived from a eukaryotic
25 organism, such as the cells derived from a mammal, especially CHO (Chinese Hamster Ovarian) cells; insect cells; cells derived from a fungus, especially a unicellular fungus or from a yeast, especially of the strain Pichia, Saccharomyces, Schizosaccharomyces and
30 most particularly selected from the group consisting of Saccharomyces cerevisiae, Schizosaccharomyces pombe, Schizosaccharomyces malidevorans, Schizosaccharomyces

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sloofiae, Schizosaccharomyces octosporus. Likewise, among the cells derived from a prokaryotic organism, there may be used, without this constituting a limitation, the cells of a strain of Escherichia coli (E coli) or enterobacterial cells. A large number of these cells are commercially available in collections, such as ATCC (Rockville, MA, USA) and AFRC (Agriculture & Food Research Council, Norfolk, UK). The cell may also be of the wild-type or mutant type. The mutations are described in the literature accessible to persons skilled in the art.

For the purposes of the present invention, an E. coli DH5a cell (marketed by the company CLONTECH under the reference: C2007-1) is used.

The expression cassette of the invention is intended for the production of the PTc100 protein or for fragments of said protein which are produced by the abovementioned E. coli cell, and which are recognized by human antisera. Such antisera are obtained from patients who have contracted a Trypanosoma cruzi infection recently or long ago, and contain immunoglobulins specifically recognizing PTc100. Of course, the PTc100 protein can also be recognized by other antibodies, such as for example monoclonal or polyclonal antibodies obtained by immunization of various species with the natural abovementioned protein, the recombinant protein or fragments or peptides thereof.

PTc100 protein is understood to mean the natural Trypanosoma cruzi cytoplasmic antigen, or the antigen produced especially by the genetic recombination techniques described in the present application, or any fragment or mutant of this antigen, provided

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that it is immunologically reactive with antibodies directed against the PTc100 protein of this parasite.

Advantageously, such a protein has an amino acid sequence having a degree of homology of at least 70%, preferably of at least 85%, and most preferably of at least 95% relative to the sequence identified in the identifier SEQ ID No.2. In practice, such an equivalent can be obtained by deletion, substitution and/or addition of one or more amino acids of the native or recombinant protein. It is within the capability of persons skilled in the art to perform, using known techniques, these modifications without affecting immunological recognition.

Within the framework of the present invention, the PTc100 protein can be modified in vitro, especially by deletion or addition of chemical groups, such as phosphates, sugars or myristic acids, so as to enhance its stability or the presentation of one or several epitopes.

The expression cassette according to the invention allows the production of a PTc100 protein (having an amino acid sequence as specified above) and fragments of said protein, fused with an exogenous element which can help its stability, its purification, its production or its recognition. The choice of such an exogenous element is within the capability of persons skilled in the art. It may be especially a hapten, an exogenous peptide or a protein.

The expression cassette according to the invention comprises the elements necessary for the expression of said DNA fragment in the cell considered. "Elements necessary for the expression" is understood

to mean the elements as a whole which allow the transcription of the DNA fragment into messenger RNA (mRNA) and the translation of the latter into protein.

The present invention also extends to a vector
5 comprising an expression cassette according to the invention. This may be a viral vector and especially a vector derived from a baculovirus, more particularly intended for expression in insect cells, or an adeno-virus-derived vector for expression in mammalian cells.

10 It may also be an autonomously replicating plasmid vector and in particular a multiplicative vector.

The present invention also relates to a cell derived from a prokaryotic or eukaryotic organism,
15 comprising an expression cassette, either in a form integrated in the cellular genome, or inserted in a vector. Such a cell was previously defined.

The subject of the present invention is also a process for preparing a PTc100 protein, or fragments of
20 said protein, according to which:

(i) a cell derived from a prokaryotic or eukaryotic organism, comprising the expression cassette according to the invention, is cultured under appropriate conditions; and

25 (ii) the expressed protein derived from the abovementioned organism is recovered.

The present invention also relates to one or more peptides, whose amino acid sequence corresponds to a portion of the sequence of the PTc100 protein and
30 exhibiting, alone or as a mixture, a reactivity with the entire sera from individuals or animals infected with Trypanosoma cruzi.

The peptides can be obtained by chemical synthesis, lyses of the PTc100 protein or by genetic recombination techniques.

The invention also relates to monoclonal or
5 polyclonal antibodies obtained by immunological reaction of a human or animal organism to an immunogenic agent consisting of the natural or recombinant PTc100 protein and fragments thereof, or of a peptide, as defined above.

10 The present invention also relates to a reagent for the detection and/or monitoring of a Trypanosoma cruzi infection, which comprises, as reactive substance, a PTc100 protein as defined above, or fragments thereof, a peptide or a mixture of peptides as
15 defined above, or at least one monoclonal or polyclonal antibody as described above.

The above reagent may be attached directly or indirectly to an appropriate solid support. The solid support may be especially in the form of a cone, a
20 tube, a well, a bead and the like.

The term "solid support" as used here includes all materials on which a reagent can be immobilized for use in diagnostic tests. Natural or synthetic materials, chemically modified or otherwise, can be
25 used as solid supports, especially polysaccharides such as cellulose-based materials, for example paper, cellulose derivatives such as cellulose acetate and nitrocellulose; polymers such as vinyl chloride, polyethylene, polystyrenes, polyacrylate or copolymers such as
30 polymers of vinyl chloride and propylene, polymers of vinyl chloride and vinyl acetate; styrene-based copoly-

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mers, natural fibers such as cotton and synthetic fibers such as nylon.

Preferably, the solid support is a polystyrene polymer or a butadiene/styrene copolymer. Advan-
5 tagedously, the support is a polystyrene or a styrene-based copolymer comprising between about 10 and 90% by weight of styrene units.

The binding of the reagent onto the solid support may be performed in a direct or indirect
10 manner.

Using the direct manner, two approaches are possible: either by adsorption of the reagent onto the solid support, that is to say by noncovalent bonds (principally of the hydrogen, Van der Waals or ionic
15 type), or by formation of covalent bonds between the reagent and the support. Using the indirect manner, an "anti-reagent" compound capable of interacting with the reagent so as to immobilize the whole onto the solid support can be attached beforehand (by adsorption or
20 covalent bonding) onto the solid support. By way of example, there may be mentioned an anti-PTc100 antibody, on the condition that it is immunologically reactive with a portion of the protein different from that involved in the reaction for recognizing the
25 antibodies in the sera; a ligand-receptor system, for example by grafting onto the PTc100 protein a molecule such as a vitamin, and by immobilizing onto the solid phase the corresponding receptor (for example the biotin-streptavidin system). Indirect manner is also
30 understood to mean the preliminary grafting or fusion by genetic recombination of a protein, or a fragment of this protein, or of a polypeptide, to one end of the

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PTc100 protein, and the immobilization of the latter onto the solid support by passive adsorption or covalent bonding of the protein or of the polypeptide grafted or fused.

5 The invention also relates to a process for the detection and/or monitoring of a Trypanosoma cruzi infection in a biological sample, such as a blood sample from an individual or an animal likely to have been infected with Trypanosoma cruzi, characterized in
10 that said sample and a reagent as defined above are placed in contact, under conditions allowing a possible immunological reaction, and the presence of an immune complex with said reagent is then detected.

By way of non-limiting example, there may be
15 mentioned the sandwich-type detection process in one or more stages, as especially described in patents FR 2,481,318 and FR 2,487,983, which consists in reacting a first monoclonal or polyclonal antibody specific for a desired antigen, attached onto a solid support,
20 with the sample, and in revealing the possible presence of an immune complex thus formed using a second antibody labelled by any appropriate marker known to persons skilled in the art, especially a radioactive isotope, an enzyme, for example peroxidase or alkaline
25 phosphatase and the like, using so-called competition techniques well known to persons skilled in the art.

The subject of the invention is also an active immunotherapeutic composition, especially a vaccinal preparation, which comprises as active ingredient, a
30 natural or recombinant PTc100 protein or fragments thereof, or the peptides identified above, the active ingredient being optionally conjugated with a pharma-

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The present invention also covers a pharmaceutical composition intended for the treatment or for the prevention of a Trypanosoma cruzi infection in man or in an animal, comprising a therapeutically effective quantity of an expression cassette, a vector, a cell derived from a prokaryotic or eukaryotic organism as defined above, a PTc100 protein according to the invention, or fragments thereof, or an antibody of the invention.

15 The term probe as used in the present invention refers to a DNA or RNA containing at least one strand having a nucleotide sequence which allows hybridization to nucleic acids having a nucleotide sequence as represented in the identifier SEQ ID No.1, or a complementary or antisense sequence, or a sequence equivalent to
20 said sequence, and especially a sequence having, for any succession of 5 to 100 contiguous monomers, at least 50%, preferably at least 60%, or even better at least 85% homology with SEQ ID No.1, with fragments
25 thereof, or with a synthetic oligonucleotide allowing such a hybridization, nonmodified or comprising one or more modified bases such as inosine, 5-methyldeoxycytidine, deoxyuridine, 5-dimethylaminodeoxyuridine, 2,6-diaminopurine, 5-bromodeoxyuridine or any other modified
30 base. Likewise, these probes may be modified at the level of the sugar, namely the replacement of at least one deoxyribose with a polyamide (P. E. NIELSEN

et al. (1991) (13)), or at the level of the phosphate group, for example its replacement with esters, especially chosen from esters of diphosphate, of alkyl and arylphosphonate and of phosphorothioate.

5 The probes may be much shorter than the sequence identified in the identifier SEQ ID No.1. In practice, such probes comprise at least 5 monomers, advantageously from 8 to 50 monomers, having a hybridization specificity, under defined conditions, to form
10 a hybridization complex with DNA or RNA having a nucleotide sequence as defined above.

A probe according to the invention can be used for diagnostic purposes as capture and/or detection probe, or for therapeutic purposes.

15 The capture probe can be immobilized on a solid support by any appropriate means, that is to say directly or indirectly, for example by covalent bonding or passive adsorption.

The detection probe is labelled by means of a
20 marker chosen from radioactive isotopes, enzymes especially chosen from peroxidase and alkaline phosphatase, and those capable of hydrolyzing a chromogenic, fluorigenic or luminescent substrate, chromophoric chemical compounds, chromogenic, fluorigenic or
25 luminescent compounds, nucleotide base analogs, and biotin.

The probes of the present invention which are used for diagnostic purposes can be used in any known hybridization techniques, and especially the so-called
30 "Dot-Blot" technique (Maniatis et al. (1982) (14)), Southern Blotting technique (Southern E. M. (1975) (15)), Northern Blotting technique, which is a

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technique identical to the Southern Blotting technique but which uses RNA as target, sandwich technique (Dunn A.R. et al. (1977) (16)). Advantageously, the sandwich tech-nique is used which comprises a specific capture
5 probe and/or a specific detection probe, it being understood that the capture probe and the detection probe must have a nucleotide sequence which is at least partially different.

Another application of the invention is a
10 therapeutic probe for treating infections due to Trypanosoma cruzi, said probe being capable of hybridizing in vivo with the DNA or RNA of the parasite to block the trans-lation and/or transcription and/or replication pheno-mena.

15 A primer is a probe comprising 5 to 30 monomers, having a hybridization specificity, under prede-fined conditions, for the initiation of an enzymatic polymerization, for example in an amplification tech-nique such as PCR (Polymerase Chain Reaction), in an
20 elongation process such as sequencing, in a reverse transcription method and the like.

A preferred probe or primer will contain a nucleotide sequence chosen from the sequences SEQ ID No.7, SEQ ID No.8, SEQ ID No.9, SEQ ID No.10, SEQ ID
25 No.12.

The invention also relates to a reagent for detecting and/or identifying Trypanosoma cruzi in a biological sample, comprising at least one probe as defined above, and in particular a capture probe and a
30 detection probe, either or both corresponding to the above definition.

004280 "SEQ ID NO. 12"

The invention therefore provides a process for selectively detecting and/or for identifying Trypanosoma cruzi in a biological sample, according to which the RNA, extracted from the parasite and optionally
5 denatured, or the DNA, denatured extract, or the DNA obtained from reverse transcription of the RNA, is exposed to at least one probe as defined above and the hybridization of said probe is detected.

The invention will be understood more clearly
10 upon reading the detailed description below which is made with reference to the accompanying figures in which:

Figure 1 represents the restriction map of the Tc100 gene, which map is deduced by Southern blotting
15 of different fragments obtained after digestion of Trypanosoma cruzi DNA with restriction endonucleases.

Figure 2 is a schematic representation of the three overlapping regions of the Tc100 cDNA. The numbered arrows represent the oligonucleotides used as
20 primers for the PCR amplification.

Example 1: Isolation of the Tc50 clone

An expression library was constructed from
25 Trypanosoma cruzi genomic DNA fragments. The T. cruzi, strain G (YOSHIDA. N, (1983) (17)), DNA isolated from the metacyclic trypomastigote stage was digested with the enzyme DNase I. After selection of the fragments according to their size, they were ligated to synthetic
30 EcoRI adaptors and cloned into the EcoRI site of lambda gtl1 vector DNA (Young and Davis, 1983 (3); Cotrim et al., 1990) (4).

The clone, called Tc50 by the applicant, was isolated from the library by immunological screening with the aid of a mixture of sera from patients suffering from the chronic phase of the Chagas disease.

5 The Tc50 phage clone was purified, amplified and the insert was detected by the PCR ("Polymerase Chain Reaction") technique with the aid of the primers:

SEQ ID No.3 5' (GGTGGCGACGACTCCTGGAGCCCG)3' 24,

and

10 SEQ ID No.4 5' (TTGACACCAGACCAACTGGTAATG)3' 24
corresponding respectively to the nucleotide sequence of the left and right arms of the lambda gt11 phage DNA.

15 The 594 base pairs (bp) Tc50 DNA fragment, after EcoRI digestion, was subcloned into the expression vector pGEX (Pharmacia) linearized with EcoRI. The sequencing of the Tc50 clone DNA was carried out in this same vector with the aid of specific primers situated in 3' and 5' of the cloning site of
20 pGEX, according to the chain termination technique (Sanger et al., 1977 (5)) and according to the manufacturer's procedure (USB-Amersham).

The nucleotide sequence of the 594 bp Tc50 fragment as well as its deduced amino acid sequence
25 (198 aa) are represented in the identifiers SEQ ID No.1 and SEQ ID No.2, respectively. The nucleotide sequence of the 594 bp Tc50 fragment starts at nucleotide (nt) 1232 and ends at nucleotide 1825. The corresponding amino acid sequence starts at amino acid 323 and ends
30 at amino acid 520 of SEQ ID No.2.

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Example 2: Expression of the Tc50 clone in Escherichia coli

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The construct pGEX-Tc50 (198 aa) synthesizes, in the bacterium DH5alpha, a protein fused with GST ("Glutathione S Transferase"), with an apparent molecular mass of 50 kDa, which is detected by SDS-PAGE polyacrylamide gel electrophoresis (SDS: sodium dodecyl sulfate) (Laemmli, 1970 (6)). The reactivity of the protein towards chagasic human sera was confirmed by the Western blotting technique (Towbin et al., 1979 (7)) with the aid of the same mixture of chronic phase chagasic sera which is used for screening the lambda gt11 library.

The soluble fraction of the recombinant GST-Tc50 protein obtained after lysis of the bacterial extracts by ultrasound was purified by affinity chromatography on a glutathione agarose column (Sigma), according to the method of Smith and Johnson, (1988) (8).

The antigenic properties of the recombinant GST-Tc50 antigen were tested by ELISA (Voller et al., 1975 (9)). For that, microtiter plates (Maxisorp (trade name), nunc) were sensitized with 100 ng/ml of GST-Tc50 antigen in 100 mM NaHCO₃ (pH 9.6). After incubation with the patients' sera, the immune complexes were detected with the aid of a peroxidase-coupled anti-human IgG goat serum.

The results are presented in the accompanying table and show that the entire chagasic human sera tested react specifically with the recombinant protein. No cross-reactivity was observed on 7 sera from

patients suffering from cutaneous or visceral leishmaniasis.

5 Example 3: Identification of the native T. cruzi protein having the antigenic determinants of the Tc50 clone

10 The detection of the native T. cruzi protein was performed after immunopurification of a mixture of chagasic human sera on the corresponding recombinant protein called PTc50 by the applicant.

15 The eluate of monospecific polyclonal antibodies which is obtained was used as probe, in Western blotting, on total protein extracts of different stages of the parasite. The selected antibodies specifically reacted with a protein of apparent molecular mass 100 kDa, called PTc100 by the applicant, which is expressed in all the tested strains of the parasite.

20 Example 4: Molecular analysis of the Tc100 gene -Southern blots

25 In order to establish the restriction map of the Tc100 gene (Figure 1), the T. cruzi, strain G, nuclear DNA was digested with different restriction endonucleases (BamHI, EcoRI, HindIII, PstI, PvuII, SacI, BamHI/EcoRI, BamHI/PvuII, EcoRI/HindIII, EcoRI/PstI, EcoRI/PvuII, EcoRI/SacI, PstI/SacI, PstI/PvuII, PvuII/SacI, PvuII/HindIII), separated on agarose gel and then transferred onto a nylon filter
30 according to the Southern technique. The Southern blot hybridization was performed with the 594 bp Tc 50 DNA, which is a fragment of the Tc100 DNA described above,

radiolabelled with ^{32}P by random incorporation (Amersham).

- Cloning of a 3500 bp Tc100 genomic fragment

5 According to the results obtained by Southern blotting, the *T. cruzi*, strain G, genomic DNA was digested with the enzyme EcoRI and then separated on agarose gel. The EcoRI restriction fragments of about 3500 bp (Figure 1) were cloned into the vector lambda
10 gt10 (Huynh et al., 1984 (10)) linearized by EcoRI. The phage clone containing the 3500 bp Tc100 genomic insert was isolated with the aid of the 594 bp radiolabelled probe described above. A 1041 bp fragment situated in
15 the 3' region of the 3500 bp Tc100 genomic insert was sequenced. This sequencing was carried out gradually with the aid of the following primers:

SEQ ID No.5 5' (TCGGGCACTGACGCGGCG) 3' 18

SEQ ID No.6 5' (CTTATGAGTATTTCTTCCAGGGTA) 3' 24

20 The primer SEQ ID No.5 is situated in the previously sequenced portion of the 594 bp Tc50 fragment. The primer SEQ ID No.6 corresponds to the lambda gt10 phage primer.

25 This 1041 bp fragment, which starts at nucleotide 1403 and ends at nucleotide 2443 of SEQ ID No.1, has an open reading frame in phase with the sequence of the 594 bp Tc50 fragment.

Example 5: Cloning of the Tc100 cDNA

The cDNA was synthesized from total RNA from *T. cruzi*, strain G, epimastigots. The Tc100 cDNA was amplified by the PCR technique in three different fragments: a fragment A corresponding to the 5' region of 1459 bp, a fragment B corresponding to the central region of 942 bp, a fragment C corresponding to the 3' region of 1406 bp of the Tc100 cDNA, as schematically represented in Figure 2.

- Cloning of fragment A of the Tc100 cDNA

The total cDNA synthesized by AMV ("avian myeloblastosis virus") reverse transcriptase, with the aid of random hexanucleotides (Boehringer Mannheim), was amplified, by PCR, using the following pair of primers:

SEQ ID No.7 5' (AACGCTATTATTAGAACAGTT)3' 21, and
SEQ ID No.8 5' (TGCAGCAGCGGCAGAAAGT)3' 18

SEQ ID No.7 corresponds to a portion of the consensus sequence of 35 nucleotides present in 5' of the mRNAs in trypanosomatids and called "spliced leader" (Parsons et al. 1984 (11)).

SEQ ID No.8 corresponds to the sequence complementary to a portion of the predetermined sequence of the 594 bp fragment, and starts at nucleotide 1442 and ends at nucleotide 1459 of SEQ ID No.1, according to the coding strand numbering.

After verification by Southern blotting with the aid of the radiolabelled 594 bp probe previously described, the 1459 bp cDNA fragment corresponding to

the 5' region of Tc100 was cloned into the plasmid called pCRII (trade name) (Invitrogen), and sequenced. The sequence represented in the identifier SEQ ID No.1 starts at nucleotide 1 and ends at nucleotide 1459.

5

- Cloning of fragment B of the Tc100 cDNA

The T. cruzi total cDNA was amplified by PCR with the aid of the primers:

SEQ ID No.9 : 5'(CAGCCGACGGTAGCTGCGTCCT)3' 22

10 and

SEQ ID No.10: 5'(ACATAATGGCCTCGTTCACAC)3' 21

The sequence ID No.9 which corresponds to a portion of the 594 bp predetermined sequence of the Tc100 gene starts at nucleotide 1266 and ends at nucleotide 1287 of SEQ ID No.1.

The sequence SEQ ID No.10 corresponds to the sequence complementary to a portion of the 1041 bp previously described sequence of the Tc100 gene. This sequence SEQ ID No.10 starts at nucleotide 2187 and ends at nucleotide 2207 of SEQ ID No.1, according to the coding strand numbering.

The fragment obtained, 942 bp in length, was cloned into the plasmid pCRII and sequenced. The sequence represented in the identifier SEQ ID No.1 starts at nucleotide 1266 and ends at nucleotide 2207.

- Cloning of fragment C of the Tc100 cDNA

In order to isolate the 3' portion of the Tc100 cDNA, the T. cruzi total cDNA was synthesized with the aid of the adaptor oligo(dT)₁₆ hybrid primer.

The 3' region of the Tc100 cDNA was amplified using the adaptor primer and the following pair of primers:

and

10

15 The sequence SEQ ID No.11 corresponds to the
arbitrary sequence of the adaptor represented in SEQ ID
No.11.

The Tc100 complete cDNA, 3402 bp in size, was
25 completely sequenced. It has a 2745 bp open reading
frame and the deduced amino acid sequence is 915. The
methionine codon is in position 266 and the stop codon
in position 3011.

The *Trypanosoma cruzi* Tc100 gene encodes the
30 new PTc100 protein of theoretical molecular mass 100
kDa.

Of course, since the DNA sequence of the gene has been fully identified, it is possible to produce the corresponding DNA solely by chemical synthesis, and then to insert the DNA into commercially available DNA
5 vectors, using known techniques from the technology relating to genetic recombination.

TABLE

Disease	Sera	OD (492nm) detection threshold = 0.320
CHAGAS DISEASE	1	1.358 (+)
	2	1.278 (+)
	3	0.328 (+)
	4	0.404 (+)
	5	1.378 (+)
	6	1.059 (+)
	7	0.895 (+)
	8	1.791 (+)
	9	1.635 (+)
	10	1.427 (+)
	11	1.009 (+)
	12	1.743 (+)
	13	0.530 (+)
	14	1.035 (+)
	15	0.461 (+)
CUTANEOUS LEISHMANIOSIS	16	0.291 (-)
VISCERAL LEISHMANIOSIS (Kala azar)	17	0.071 (-)
	18	0.081 (-)
	19	0.279 (-)
	20	0.098 (-)
	21	0.067 (-)
	22	0.125 (-)

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: PARANHOS-BACCALA, Glaucia
LESENECHAL, Mylene
JOLIVET, Michel
- (ii) TITLE OF INVENTION: NEW TRYPANOSOMA CRUZI ANTIGEN, AND
GENE ENCODING THE LATTER; THEIR APPLICATION TO THE
DETECTION OF CHAGAS DISEASE
- (iii) NUMBER OF SEQUENCES: 13
- (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Oliff & Berridge
(B) STREET: 700 South Washington Street, Suite 300
(C) CITY: Alexandria
(D) STATE: Virginia
(E) COUNTRY: U.S.A.
(F) ZIP: 22314
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/480,917
(B) FILING DATE: 07-JUN-1995
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Berridge, William P.
(B) REGISTRATION NUMBER: 30,024
(C) REFERENCE/DOCKET NUMBER: WPB 36400
- (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 703-836-6400
(B) TELEFAX: 703-836-2787

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3402 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AACGCTATTA TTAGAACAGT TTCTGTACTA TATTGTCATT TGGGGAGGGG GGAAAGGGGG	60
GAAGTACTTG CCGTTTTGTG TGGGTGACGA GACAACACAC ATCGAGCGGG AAGAAAAAAA	120
AAAAGGAAAT AAATTAAATT AAATTATTTG TTCTTTGAAT AGGCAAAGAA GAAGAAGAAG	180

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052300 523000
AAAAGGTGCG GGGGAGGGAG GAGAAAGCGA CACACACACA AAAAAAAAAA AAGGAATTGC 240
GGAAATAACA ACGCAAGGCG CGGACATGAC CGTGACGGTG GATTTGTTCA ATCATGCGAA 300
GCCGAGCAAC AATGAGGGCC GCGTGTGGTC TGTGGACGCC GCGACATTTA ACGAGGTGCC 360
TGAGGCGCAG CGTGTGCTGG CGGATTCGCA GTTTTATCTT GCCTACACCA TGAAGCGGCG 420
TCACGTGCTG CGTGTGGTGA AGCGCTCGAA CCTTTTGAAG GGCACCGTGC GGGCACACTC 480
AAAGCCCATT CATGCGGTGA AGTTTGTGAA TTACCGCAGT AACGTCGCAG CATCGGCTGG 540
GAAGGGGGAG TTCTTCGTGT GGGTTGTGAC GGATGAAACG GAGGCGAGCA ACGGCAAGCC 600
GGATCTCGCA GCGCGCCTCA CAGTGAAGGT GTACTTTAAG CTTCAGGATC CTGTCACAAT 660
TCCATGCTTT TCTTTCTTTA TCAACGCCGA GAGTCAGCGG CCTGATCTGC TTGTCCTTTA 720
CGAAACGCAG GCGGCAATTC TTGACAGCTC CTCCCTCATT GAGCGCTTTG ACGTGGAATC 780
ACTGGAGGCA ACACTACAGC GGAATTGCAC AACCCTGCGA ACCCTGACTC AACCGGTTAG 840
TGAGAACAGT TTATGCTCCG TTGGCTCTGG CGGATGGTTC ACCTTTACCA CGGAACCAAC 900
AATGGTAGCG GCATGCACAT TACGAAACCG CAGCACTCCA TCATGGGCGT GTTGCGAGGG 960
TGAGCCAGTG AAGGCATTGC ATCTCCTTGA CGCAACCGTT GAGGAAAATG TCAGTGTTCT 1020
CGTGGCCGCA TCTACAAAAG GGGTGTACCA ATGGCTCCTT ACGGGTGTAG CAGAACCAAA 1080
CTTGTTGCGC AAGTTTGTCA TTGATGGATC TATTGTGCGG ATGGAAAGCT CACGAGAAAC 1140
GTTTGCCGTG TTTGACGACA GGAAGCAGCT GGCGCTGGTC AACATGCATT CCCCTCATAA 1200
CTTTACCTGC ACACACTACA TGATGCCTTG TCAGGTACAG CGTAACGGCT TTTGCTTCAA 1260
TCGTACAGCC GACGGTAGCT GCGTCCTGGC TGACATGTCG ATTCGATTGA CGATCTTCCA 1320
TCTCCGGTCC TCCCGCAGGG AAGAACAGCA GCCAGGCCAA AAAACATCGG TAGTGGCGAC 1380
GGCGAAACCG GGGTGTGTGT CCTCGGGCAC TGACGCGGCG AGTAGCAGTC ATACCAATAC 1440
GACTTCTGCC GCTGCTGCAT CCCCTGCATC ACCCCCTGTT TCAGCGCCAG CCAAGGCAGC 1500
CGCGCCTCCT GCCGCGGCGC GATCGGCTGA GCCGCACGTG GGGAGCAAGA TCATTGCTAA 1560
TCTAGTGAAT CAGCTGGGGA TTAATGTCAC CCAAAGGAGC GTCGTCAGCA CTGGAGCGCC 1620
GGCCACGACG AGGTCTACGG CGGTGACGTC CACGACTACC GCGCCGAGC GAACAAGTCC 1680
ATACGGGCAC AATGGCCGAC CTGTGACGGC TGGATTGGTG GCAGCTAATA GTGGTGCCAG 1740
CGCGGCCTCG TCTCCACAG CCGCGGCGAA ACCAACAGGA GAAGAAAAGG CCTCCGCGGC 1800
ATGTGAAACG AGCTCCGTGG CGATAAATGC GACACGCCCG GCGCTTCACA ACGCCTCTCT 1860
CCCGCAGGCG CCAACGGATG GCGTTTTGGC GGCAGCAGTA TACCAGTCGG AGGGCGAGGT 1920
TCATCAGTCG CTGGAGCGGC TGGAGTCCGT CATAACCAAC ACGTCTCGGG TTCTGAAGTT 1980
GCTCCCTGAC ACCATTCGAA GAGACCATGA ACAACTTCTG AATCTGGGTT TAGAGGCACA 2040

GATGACAGAG CTGCAGCAGA GCCGTCCAAC ACCGCAAACA CAGCCGAGAG ACACAAGCTC 2100
 CGCGAAATCA TCCGTGTTTG AGACGTACAC CCTTGTTCTC ATTGCGGATT CCCTCTCTCG 2160
 CAACATCACG AAGGGGGTGA AGCGTGGTGT GAACGAGGCC ATTATGTTGC ATCTCGACCA 2220
 TGAGGTGCGG CACGCCATAG GGAACCGGCT TCGGCAAACA CAAAAGAACA TCATCAAGAG 2280
 CCGCCTCGAT GAAGCGTTGA AGGAAAGCAC TACACAGTTT ACGGCTCAAT TGACGCAAAC 2340
 GGTGGAGAAT CTGGTGAAGC GCGAGCTTGC CGAGGTGCTT GGTAGCATCA ACGGCTCCCT 2400
 CACTTCTCTC GTGAAGGAAA ATGCCTCATT ACAGAAAGAG TTGAATTCCA TAATGTCTAG 2460
 TGGGGTGTTG GATGAAATGC GTCGTATGCG GGAAGAGCTG TGCACATTGC GAGAGTCCGT 2520
 TGCGAAGCGG AAGGCAACAA TGCCAGATTC TTCTCTTCAC GCCACGAGCT CCTTTCAAGG 2580
 AAGAAGGTCT GCGCCCGAGA CAATTCTTGC AACC GCGTTA TCGATGGTGC GAGAGCAGCA 2640
 ATACCGTCAG GGA CTGGAAT ACATGTTGAT GGCTCAGCAG CCCTCTCTCC TCCTGCGGTT 2700
 CCTCAGCATA CTTACAAGGG AAAACGAAAA CGCCTACTCG GAACTTATTG AAAATGTAGA 2760
 GACGCCGAAT GACGTGTGGT GTTCGGTTCT GTTGCAACTC ATAGAGGCCG CGGCGACCGA 2820
 GGCTGAGAAG GAGGTGGTTG TTGGCGTCGC CATTGATATT CTCTCCGAGC GCGATCAAAT 2880
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 GGCAAGGTCG GAGACAACGA GCAGGTCATT CTTGCAATGC CTGAAGAACC TGGAAAAGCT 3000
 TCTGCAATCA TGATAATAAA AAGAACTCAA CGAATACAGT TGTTGATTAT TAAGGAAGGG 3060
 AAAAGAGAGA AAGAGAGAGA GAGAGAGAGA AATGTAATGG GCGTTTAGTT ACGGTAGAAA 3120
 GAAAACGTGT GGATAAGAAG GAGGGGTTTT GTGTGCGACC AGGAATTACT GGGGAACGCT 3180
 GCTACACGGC GGAATCGACC ATTTTATTAT TATTATTATT GTCTTTAGTA TTATGTTTTT 3240
 TCTTGTGTGT GTGTGTGTGT GTTTGTGTGT GTGCGGTTAT TTTGTATCCG TTTGCTCCCC 3300
 CCCCTGCCCC CCATCACCCG AGGAGAAAGT AGAATAAGAC ACATACGATT GTTGTTTTTG 3360
 TTATCCTTAA AAGGAAGAGA GACCAAAAAA AAAAAAAAAA AA 3402

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 915 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "protein"

Met 1	Thr	Val	Thr	Val 5	Asp	Leu	Phe	Asn	His 10	Ala	Lys	Pro	Ser	Asn 15	Asn
Glu	Gly	Arg	Val 20	Trp	Ser	Val	Asp	Ala 25	Ala	Thr	Phe	Asn	Glu 30	Val	Pro
Glu	Ala	Gln 35	Arg	Val	Leu	Ala	Asp 40	Ser	Gln	Phe	Tyr	Leu 45	Ala	Tyr	Thr
Met 50	Lys	Arg	Arg	His	Val	Leu 55	Arg	Val	Val	Lys	Arg 60	Ser	Asn	Leu	Leu
Lys 65	Gly	Thr	Val	Arg	Ala 70	His	Ser	Lys	Pro	Ile 75	His	Ala	Val	Lys	Phe 80
Val	Asn	Tyr	Arg	Ser 85	Asn	Val	Ala	Ala	Ser 90	Ala	Gly	Lys	Gly	Glu 95	Phe
Phe	Val	Trp	Val 100	Val	Thr	Asp	Glu	Thr 105	Asp	Ala	Ser	Asn	Gly 110	Lys	Pro
Asp	Leu	Ala 115	Ala	Arg	Leu	Thr	Val 120	Lys	Val	Tyr	Phe	Lys 125	Leu	Gln	Asp
Pro 130	Val	Thr	Ile	Pro	Cys	Phe 135	Ser	Phe	Phe	Ile	Asn 140	Ala	Glu	Ser	Gln
Arg 145	Pro	Asp	Leu	Leu 150	Val	Leu	Tyr	Glu	Thr	Gln 155	Ala	Ala	Ile	Leu	Asp 160
Ser	Ser	Ser	Leu 165	Ile	Glu	Arg	Phe	Asp 170	Val	Glu	Ser	Leu	Glu	Ala 175	Thr
Leu	Gln	Arg	Asn 180	Cys	Thr	Thr	Leu	Arg 185	Thr	Leu	Thr	Gln	Pro 190	Val	Ser
Glu	Asn	Ser 195	Leu	Cys	Ser	Val	Gly 200	Ser	Gly	Gly	Trp	Phe 205	Thr	Phe	Thr
Thr 210	Glu	Pro	Thr	Met	Val	Ala 215	Ala	Cys	Thr	Leu	Arg 220	Asn	Arg	Ser	Thr
Pro 225	Ser	Trp	Ala	Cys	Cys 230	Glu	Gly	Glu	Pro	Val 235	Lys	Ala	Leu	His	Leu 240
Leu	Asp	Ala	Thr 245	Val	Glu	Glu	Asn	Val	Ser 250	Val	Leu	Val	Ala	Ala 255	Ser
Thr	Lys	Gly	Val 260	Tyr	Gln	Trp	Leu	Leu 265	Thr	Gly	Val	Ala	Glu 270	Pro	Asn
Leu	Leu	Arg 275	Lys	Phe	Val	Ile	Asp 280	Gly	Ser	Ile	Val	Ala 285	Met	Glu	Ser
Ser 290	Arg	Glu	Thr	Phe	Ala	Val 295	Phe	Asp	Asp	Arg	Lys 300	Gln	Leu	Ala	Leu
Val 305	Asn	Met	His	Ser	Pro 310	His	Asn	Phe	Thr	Cys 315	Thr	His	Tyr	Met	Met 320

Pro	Cys	Gln	Val	Gln	Arg	Asn	Gly	Phe	Cys	Phe	Asn	Arg	Thr	Ala	Asp
				325					330					335	
Gly	Ser	Cys	Val	Leu	Ala	Asp	Met	Ser	Asn	Arg	Leu	Thr	Ile	Phe	His
			340					345					350		
Leu	Arg	Cys	Ser	Arg	Arg	Glu	Glu	Gln	Gln	Pro	Gly	Gln	Lys	Thr	Ser
		355					360					365			
Val	Val	Ala	Thr	Ala	Lys	Pro	Gly	Cys	Val	Ser	Ser	Gly	Thr	Asp	Ala
	370					375					380				
Ala	Ser	Ser	Ser	His	Thr	Asn	Thr	Thr	Ser	Ala	Ala	Ala	Ala	Ser	Pro
385				390						395					400
Ala	Ser	Pro	Pro	Val	Ser	Ala	Pro	Ala	Lys	Ala	Ala	Ala	Pro	Pro	Ala
				405					410					415	
Ala	Ala	Arg	Ser	Ala	Glu	Pro	His	Val	Gly	Ser	Lys	Ile	Ile	Ala	Asn
			420					425					430		
Leu	Val	Asn	Gln	Leu	Gly	Ile	Asn	Val	Thr	Gln	Arg	Ser	Val	Val	Ser
		435					440					445			
Thr	Gly	Ala	Pro	Ala	Thr	Thr	Arg	Ser	Thr	Ala	Val	Thr	Ser	Thr	Thr
	450					455					460				
Thr	Ala	Pro	Gln	Arg	Thr	Ser	Pro	Tyr	Gly	His	Asn	Gly	Arg	Pro	Val
465					470					475					480
Thr	Ala	Gly	Leu	Val	Ala	Ala	Asn	Ser	Gly	Ala	Ser	Ala	Ala	Ser	Ser
				485					490					495	
Pro	Thr	Ala	Ala	Ala	Lys	Pro	Thr	Gly	Glu	Glu	Lys	Ala	Ser	Ala	Ala
			500					505					510		
Cys	Glu	Thr	Ser	Ser	Val	Ala	Ile	Asn	Ala	Thr	Arg	Pro	Ala	Leu	His
		515					520					525			
Asn	Ala	Ser	Leu	Pro	Gln	Ala	Pro	Thr	Asp	Gly	Val	Leu	Ala	Ala	Ala
	530					535					540				
Val	Tyr	Gln	Ser	Glu	Gly	Glu	Val	His	Gln	Ser	Leu	Glu	Arg	Leu	Glu
545					550					555					560
Ser	Val	Ile	Thr	Asn	Thr	Ser	Arg	Val	Leu	Lys	Leu	Leu	Pro	Asp	Thr
				565					570					575	
Ile	Arg	Arg	Asp	His	Glu	Gln	Leu	Leu	Asn	Leu	Gly	Leu	Glu	Ala	Gln
			580				585						590		
Met	Thr	Glu	Leu	Gln	Gln	Ser	Arg	Pro	Thr	Pro	Gln	Thr	Gln	Pro	Arg
		595					600					605			
Asp	Thr	Ser	Ser	Ala	Lys	Ser	Ser	Val	Phe	Glu	Thr	Tyr	Thr	Leu	Val
	610					615					620				
Leu	Ile	Ala	Asp	Ser	Leu	Ser	Arg	Asn	Ile	Thr	Lys	Gly	Val	Lys	Arg
625					630					635					640
Gly	Val	Asn	Glu	Ala	Ile	Met	Leu	His	Leu	Asp	His	Glu	Val	Arg	His
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Ala	Ile	Gly	Asn	Arg	Leu	Arg	Gln	Thr	Gln	Lys	Asn	Ile	Ile	Lys	Ser
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	690					695					700				
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				725					730					735	
Glu	Met	Arg	Arg	Met	Arg	Glu	Glu	Leu	Cys	Thr	Leu	Arg	Glu	Ser	Val
			740					745					750		
Ala	Lys	Arg	Lys	Ala	Thr	Met	Pro	Asp	Ser	Ser	Leu	His	Ala	Thr	Ser
		755					760					765			
Ser	Phe	Gln	Gly	Arg	Arg	Ser	Ala	Pro	Glu	Thr	Ile	Leu	Ala	Thr	Ala
	770					775					780				
Leu	Ser	Met	Val	Arg	Glu	Gln	Gln	Tyr	Arg	Gln	Gly	Leu	Glu	Val	Met
785					790					795					800
Leu	Met	Ala	Gln	Gln	Pro	Ser	Leu	Leu	Leu	Arg	Phe	Leu	Ser	Ile	Leu
				805					810					815	
Thr	Arg	Glu	Asn	Glu	Asn	Ala	Tyr	Ser	Glu	Leu	Ile	Glu	Asn	Val	Glu
			820					825					830		
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		835					840					845			
Ala	Ala	Thr	Glu	Ala	Glu	Lys	Glu	Val	Val	Val	Gly	Val	Ala	Ile	Asp
	850					855					860				
Ile	Leu	Ser	Glu	Arg	Asp	Gln	Ile	Ala	Gln	Asn	Gly	Ala	Leu	Gly	Ser
865					870					875					880
Lys	Leu	Thr	Thr	Ala	Met	Arg	Ala	Phe	Glu	Arg	Gln	Ala	Arg	Ser	Glu
				885					890					895	
Thr	Thr	Ser	Arg	Ser	Phe	Leu	Gln	Cys	Leu	Lys	Asn	Leu	Ile	Lys	Leu
			900					905					910		
Leu	Gln	Ser													
		915													

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "phage DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGTGGCGACG ACTCCTGGAG CCCG

24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "phage DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTGACACCAG ACCAACTGGT AATG

24

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCGGGCACTG ACGCGGCG

18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "phage lambda gt10 DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTTATGAGTA TTTCTTCCAG GGTA

24

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

00130735-002400
064280-554860

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AACGCTATTA TTAGAACAGT T

21

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGCAGCAGCG GCAGAAAGT

18

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CAGCCGACGG TAGCTGCGTC CT

22

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

09130735-002499
1864280-52495760

ACATAATGGC CTCGTTTACA C

21

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GACTCGCTGC AGATCGATTT TTTTTTTTTT TTTT

34

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGAAGAGACC ATGAACAACT T

21

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GACTCGCTGC AGATCGAT

18

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CLAIMS

1. A synthetic or isolated nucleic acid fragment which comprises a nucleotide sequence that is identical, complementary, antisense or equivalent to a first
5 sequence starting at nucleotide 1232 and ending at nucleotide 1825 of SEQ ID NO:1.

2. The nucleic acid fragment according to claim 1, wherein said nucleotide sequence is identical, complementary, antisense or equivalent to a second
10 sequence starting at nucleotide 1232 and ending at nucleotide 2207 of SEQ ID NO:1.

3. The nucleic acid fragment according to claim 1, wherein an at least 30 nucleotide segment of said nucleotide sequence is at least 50% homologous with a
15 correspondingly long segment of the sequence identified in SEQ ID NO:1.

4. The nucleic acid fragment according to claim 2, wherein an at least 30 nucleotide segment of said nucleotide sequence is at least 50% homologous with a
20 correspondingly long segment of the sequence identified in SEQ ID NO:1.

5. A probe for identifying Trypanosoma cruzi, said probe comprising a nucleotide sequence that is hybridizable to at least a segment of a nucleic acid
25 according to claim 1.

6. The probe according to claim 5, wherein said probe comprises 5 to 100 nucleotides.

7. The probe according to claim 5, wherein said probe comprises 8 to 50 nucleotides.

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8. A primer for amplifying a nucleotide sequence, said primer comprising a nucleotide sequence that allows hybridization to at least a segment of a nucleic acid according to claim 1.

5 9. The primer according to claim 8, wherein said nucleotide sequence comprises at least five nucleotides.

10 10. The primer according to claim 9, wherein said nucleotide sequence is selected from the group consisting of SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10 and SEQ ID NO:12.

15 11. A reagent for detecting or identifying Trypanosoma cruzi in a biological sample, said reagent comprising at least one of a capture probe and a detection probe, wherein said capture probe and said detection probe each comprise a nucleotide sequence that is hybridizable to at least a segment of a nucleic acid according to claim 1, and wherein said capture probe and said detection probe, if they are both present, have nucleotide sequences that are at least partially different from one another.

12. The reagent according to claim 11, wherein said capture probe is attached to a solid support.

13. The reagent according to claim 12, wherein said capture probe is directly attached to said solid support.

25 14. The reagent according to claim 12, wherein said capture probe is indirectly attached to said solid support.

15. The reagent according to claim 11, wherein said detection probe is labelled by a marker selected from the

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group consisting of radioactive isotopes, enzymes capable of hydrolyzing a chromogenic, fluorigenic or luminescent substrate, chromophoric chemical compounds, fluorigenic compounds, luminescent compounds, nucleotide base
5 analogs, and biotin.

16. The reagent according to claim 15, wherein said enzymes are selected from the group consisting of peroxidase and alkaline phosphatase.

17. The reagent according to claim 11, comprising
10 at least one primer comprising a nucleotide sequence that allows hybridization to at least a segment of a nucleic acid which comprises a nucleotide sequence that is identical, complementary, antisense or equivalent to a first sequence starting at nucleotide 1232 and ending at
15 nucleotide 1825 of SEQ ID NO:1.

18. A method for detection and/or identification of Trypanosoma cruzi in a biological sample, comprising exposing to at least one probe according to claim 5 denatured DNA extracted from Trypanosoma cruzi or DNA
20 obtained by reverse transcription of RNA extracted from Trypanosoma cruzi; and detecting hybridization of said probe.

19. A method for detection and/or identification of Trypanosoma cruzi in a biological sample, comprising
25 exposing extracted RNA from Trypanosoma cruzi to at least one probe according to claim 5; hybridizing said probe with said RNA; and detecting said hybridization.

20. The method according to claim 18, wherein before said DNA is exposed to said probe, said DNA is

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amplified in the presence of an enzymatic system with at least one primer, wherein said primer comprises a nucleotide sequence that is hybridizable to a nucleic acid sequence that is identical, complementary, antisense or equivalent to a sequence identified in SEQ ID NO:1.

ABSTRACT OF THE DISCLOSURE

The nucleotide sequence of Tc100, a gene encoding PTc100, a new Trypanosoma antigen, and the amino acid sequence of PTc100 are described. Tc100 and PTc100, or
5 fragments thereof, modified or otherwise, can be used directly or indirectly for the detection of Trypanosoma cruzi, or for the monitoring of the infection generated by Trypanosoma cruzi, in man or in animals.

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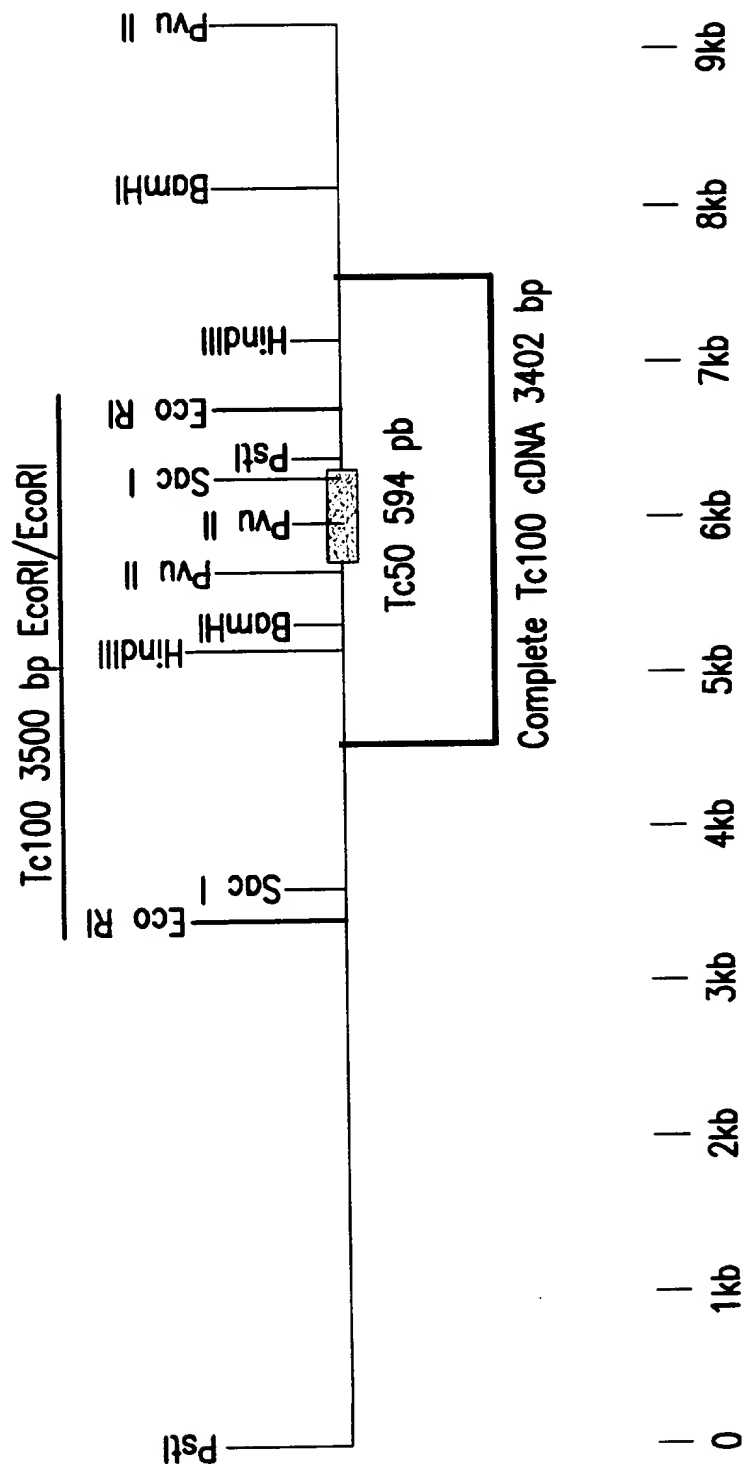


FIG.1

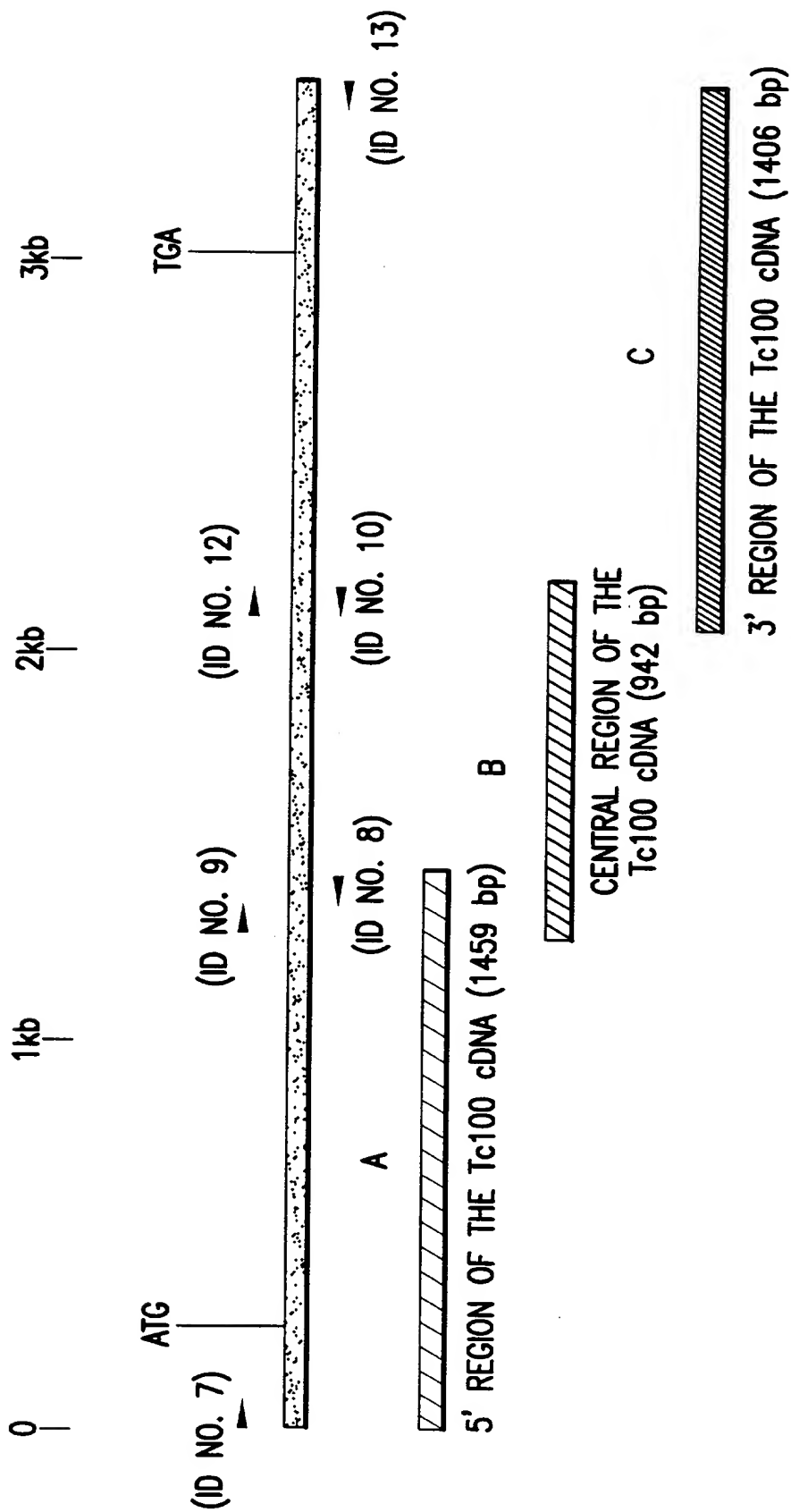


FIG.2

APPLICATION FOR UNITED STATES PATENT DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: NEW TRYPANOSOMA CRUZI ANTIGEN, GENE ENCODING THEREFOR AND METHODS OF DETECTING AND TREATING CHAGAS DISEASE

described and claimed in the specification:

Check one

- *a. ☐ attached hereto.
b. ☒ filed on June 7, 1995 as Application No. 08/480,917 and amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

Under Title 35, U.S. Code §119, the priority benefits of the following foreign application(s) and/or United States provisional application(s) filed within one year prior to this application are hereby claimed:

French Patent Application No. 94 10132, filed August 12, 1994

The following application(s) for patent or inventor's certificate on this invention were filed in countries foreign to the United States of America either (a) more than one year prior to this application, or (b) before the filing date of the above-named foreign priority application(s) and/or United States provisional application(s):

I hereby appoint the following as my attorneys of record with full power of substitution and revocation to prosecute this application and to transact all business in the Patent Office:

**James A. Oliff, Reg. No. 27,075; William P. Berridge, Reg. No. 30,024;
Kirk M. Hudson, Reg. No. 27,562; Thomas J. Pardini, Reg. No. 30,411;
Edward P. Walker, Reg. No. 31,450; Robert A. Miller, Reg. No. 32,771 and
Mario A. Costantino, Reg. No. 33,565.**

ALL CORRESPONDENCE IN CONNECTION WITH THIS APPLICATION SHOULD BE SENT TO OLIFF & BERRIDGE, PLC, P.O. BOX 19928, ALEXANDRIA, VIRGINIA 22320, TELEPHONE (703) 836-6400.

I hereby declare that I have reviewed and understand the contents of this Declaration, and that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1 Typewritten Full Name of First or Sole Inventor

2 **Inventor's Signature:

3 **Date of Signature:

	Given Name	Middle Initial	Family Name
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	Month	Day	Year
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	City	State or Province	Country
Citizenship:	Brazil and Switzerland		
	Post Office Address:		
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		69003 Lyon, FRANCE	

*If Box (a.) is checked, this form may be executed only when attached to the specification (including claims).

**Note to Inventor: Please sign name exactly as it appears above and insert actual date of signing.

IF THERE IS MORE THAN ONE INVENTOR USE PAGE 2 AND PLACE AN "X" HERE ☒

(Discard this page in a sole inventor application)

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3 Date of Signature
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3 Date of Signature
Residence City State or Province Country
Citizenship
Post Office Address
(Insert complete mailing address, including country)

Note to Inventor: Please sign name on line 2 exactly as it appears in line 1 and insert the actual date of signing on line 3.

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